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Federal Water Pollution Control Administration
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An Electron Microscope Study of Colloids in Waste Water

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■ Electron micrographs of the colloidal fraction in the effluent from an activated sludge waste water-treatment plant show fragments of bacterial cell walls as the dominant material. Viruses, phage, flagella, and other cellular debris are present to a lesser degree. The cell wall fragments appear to have a thickness near 100 A. and a width from 500 to 5000 A. (0.05 to 0.5 micron). The fragments are loosely clumped together in preparations which have been freeze-dried at −75° C. but are flattened out and almost invisible in preparations dried near room temperature. Clarification by membrane filtration, or by flocculation with lime or ferric chloride, removes most of the colloidal material of cellular origin.

The colloidal fraction of waste waters from sewage treatment plants has not previously been observed directly. Bishop *et al.* (1965, 1967) have demonstrated that colloidal organic matter will not be removed on columns of activated carbon in reasonable periods of time. Organic material associated with previous use of waste water must be removed before the water can be judged fit for intentional re-use. The only materials of colloidal dimensions previously identified in treated waste water are viruses and humic substances (Clarke and Kabler, 1964; Wedgwood, 1952). Rudolfs and Balmat (1952) made electron micrographs of the colloids in raw sewage but do not appear to have used freeze-drying. It has been assumed that cellular debris will be present, but its quantity and structure have been strictly matters for conjecture. After this work was completed, Rickert and Hunter (1966) reported their work on hign speed centrifugation of sewage effluents.

The authors have made electron microscope observations of the colloidal constituents of waste water from a modern activated sludge treatment plant using low temperature freeze-drying to preserve the structure (Claesson *et al.*, 1967). Solid matter in the size range from 0.01 to 1 micron was found to fall into two distinct groups: bacteria, viruses, phages, and related compact bodies having a definite size and shape; and shapeless fragmentary material loosely clumped together. The principal component of this fragmentary group appears to be cell walls from bacteria, although other cellular debris is undoubtedly present. The aggregation of cell wall material is certainly produced in part by the operations necessary to prepare the specimens. However, the loose open nature of the fragmentary material demonstrates that the particles are not strongly flocculated in the starting material.

Technique

Secondary effluent was obtained from the Uppsala, Sweden, sewage treatment plant which was completed in 1957 and is currently being doubled in capacity. This is an activated sludge plant using step aeration. Operating difficulties have been minimal despite overloading and the presence of branched-chain alkylbenzene sulfonates (ABS) in the detergents. Effluent BOD averages 30 mg. per liter, corresponding to a removal efficiency of 88%.

In this work, effluent was immediately filtered through glass fiber filter mat 1106 BH (Mine Safety Appliances, Pittsburgh, Pa.) to remove suspended matter. A separate sample of this filtered product was sealed under vacuum and heated in boiling water for 1 hour before shipment to Cincinnati for chemical analysis (Table I). The total organic carbon (TOC) of 11 mg. per liter on the filtered material corresponds to about 30 mg. per liter as COD and about 20 mg. per liter as BOD (Williams, 1966). Uppsala has very hard water, ranging from 15 to 20 German degrees, corresponding to 5.3 to 7.1 meq. per liter with an alkalinity of 4.5 to 5.0 meq. per liter. The hardness of the effluent is correspondingly high. Total dissolved solids (TDS) may be estimated as about 490 mg. per liter based on the conductivity of 750 μmhos.

Electron microscopic examination of organic colloids must be carried out on dry specimens. It is necessary to separate the colloids, which probably do not exceed 5 mg. per liter, from the large excess of dissolved salts which would otherwise obscure the field. Colloids were concentrated by centrifugation

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Table I. Analysis of Filtered Secondary Effluent at Uppsala Sewage Treatment Plant

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	Mg./Liter	Meq./Liter
ABS (MBAS) detergents	2.0	...
Ammonia nitrogen	18.6	1.33
Nitrate nitrogen	0.6	...
Nitrite nitrogen	0.4	...
Bicarbonate	361.0	5.92
Calcium	94.0	4.70
Magnesium	12.1	0.99
Organic carbon	11.4	...
Conductivity	... 730 μmhos	...

in a Spinco Model L centrifuge with swinging buckets at 20,000 r.p.m., corresponding to an acceleration of 45,000 G. at the bottom of the buckets 10 cm. from the axis of rotation. This treatment would bring down most of the colloids. However, Rickert and Hunter (1966) found that even 62,500 G was insufficient to remove entirely the true colloidal fraction down to 10 A. A small quantity of soft solids was collected at the bottom of the 30-ml. cups after centrifugation for 20 minutes. The supernatant was poured off, leaving 1 to 2 ml. of fluid in the bottom. The solids were dispersed in double-distilled water using a low power ultrasonic cleaning bath to agitate the tube. Centrifugation and decantation were repeated three times. Volumes were made twice with double-distilled water, giving an estimated dilution factor for soluble material (nonsedimenting) of about 1 to 400. The dilution factor varied between preparations; its effectiveness was made obvious by the absence of dense salt crystals in the micrographs.

Centrifugation in large tubes, even in swinging buckets, is hampered by unavoidable convection. To eliminate convection, capillary centrifuge tubes, 1.5 mm. in internal diameter and 7 cm. long, were made from lime-soda glass tubing and were supported in transparent plastic inserts machined to fit the centrifuge buckets. The inner well of the support was intentionally oversize, so that the glass capillaries could be supported in water. The only breakage occurred when one of the support wells was allowed to run dry. New capillaries were used for each run, usually in triplicate. Supernatant fluid was decanted with a fine-bore glass tube and wash water was introduced in the same way. The contents of the tube were mixed by inserting a fine glass fiber with a 1-mm. ball at its end and pumping this ball up and down about 20 times. Tests with ink and water proved that capillary tubes of this size eliminate convection and that the mixing technique was adequate. Samples of the final centrifugate were taken by cutting the tube about 3 mm. above the bottom and drawing up the residue in a fine glass capillary tube.

Previous work (Claesson *et al.*, 1967) had shown that soft colloidal matter can best be seen if the specimens are freeze-dried and shadowed directly on the supporting film. Surface tension forces in evaporating drops exert enormous pressures on small particles. A sphere 1 micron in diameter will be squeezed with a pressure of about 3 atmospheres as water evaporates around it. This pressure flattens all loose aggregates and may even produce artifacts.

Specimens were freeze-dried by spraying onto an evaporated carbon film which was held at −130° C. (Claesson *et al.*, 1967). Individual droplets were about 200 to 600 microns in diameter. The frozen droplets were evaporated at −75° C. All freeze-dried specimens were prepared on carbon films which had been evaporated on freshly cleaved mica. Some specimens which were evaporated above the freezing point were made on cellulose nitrate films. A thicker layer of water was applied to slides that were not freeze-dried. The dried specimens were shadowed with 80% platinum, 20% palladium alloy at −60° to −30° C. without removal from the drying chamber. The cotangent of the shadow angle was approximately 3, giving shadows which are about three times as long as the height of the object. The dried and shadowed films were floated off their supports onto clean water and were picked up on electron microscope grids. Micrographs were made in a Siemens Elmiscope No. 1 accelerated with 60 kv. Magnification was 20,000 times as measured on the glass photographic plate. Ilford N40 process plates were used.

Clarification with lime or ferric chloride was carried out on a custom-built gang stirrer having conventional geometry. Rapid mix at 100 r.p.m. was continued for one minute after the last reagent was added. Slow mix at 20 to 22 r.p.m. was employed for 15 to 30 minutes, followed by 30 to 60 minutes settling. Supernatant fluid was decanted and filtered through a glass fiber filter mat before centrifuging.

Although the resolving power of the electron microscope under optimum conditions is more than 10 A. units, the useful resolving power is set by the techniques necessary to produce sufficient contrast in the object. Compounds of carbon, hydrogen, oxygen, and nitrogen produce insufficient contrast to be visible if their thickness is less than a few hundred Angstrom units. These compounds can be "seen" by evaporating metal atoms against them from a low angle. This produces a shadow where no metal had reached the supporting film as well as a coating of metal atoms on the opposite side of the object. Since the metal atoms agglomerate into grains, the ultimate resolution is fixed by the size of metallic grains. In the current work at a plate magnification of 20,000, the average diameter of the grains of shadowing metal is about 0.07 mm. on the plate. When shadowing is done at a low angle (cotangent about 3) thinner threadlike objects can be distinguished if they are long enough. This sets the ultimate resolution at 30 A. (3 mμ). Fibers of one grain wide with a shadow only 90 A. wide are clearly visible in some preparations (Figure 1) but may not show in half-tone reproductions. These fibers, which might be nucleic acids, are relatively infrequent and do not account for a significant fraction of the colloidal matter. They are useful as indicators of the resolution, even though they are difficult to see on the fluorescent screen and can only be studied on photographic plate.

Bacteria are the principal recognizable particles at low magnifications in specimens which have not been freeze-dried. Microbial cell walls or ghosts are also abundant. Shadow lengths show double cell walls with an apparent thickness of about 400 A. Partially emptied bacterial cells as in Figure 1 are presumably spores (Scanga, 1964). Virus and phage particles are also recognizable by their uniform sizes and distinctive shapes. It is, of course, impossible to identify species or strains from electron micrographs alone.

In the specimens which have been dried without freezing, there are traces of fragmentary organic matter as thin as or

thinner than cell walls. The fragmentary fraction casts very little shadow and has a low density in the electron beam. This amorphous fraction shows gradations from obvious bacterial ghosts through partially distintegrated cell walls to thin irregular pieces only a few Angstroms across. Some cell wall fragments show the characteristic polygonal structure reported by Houwink (1963) (Figure 2). The fragmentary material may not be solely cell walls, since other cellular structures are expected. However, there is very little material, other than viruses and whole bacteria, which might not have come from partially disintegrated cell walls.

Figure 3 shows at low magnification a preparation that is rich in virus-like particles. This preparation was centrifuged in capillary tubes, and the specimen was taken from the bottom of the tube. All of the above plates were obtained without freeze-drying. Relatively thicker layers of liquid can be evaporated in this way producing dense populations of bacteria and viruses. However, fragmentary matter is largely obscured as flat shadowless traces, which can barely be distinguished in Figures 1 through 3.

Freeze-drying produced large loose clumps of amorphous solids having a structure quite different from the flat appearance of preparations which had been evaporated as a liquid. A typical aggregate is shown in Figure 4. In this figure, fragments of cell wall are associated with other unidentifiable debris. Figure 5 shows the remains of a cell wall that has not been flattened by surface tension forces. In Figure 6, the principal structure appears to be derived from a cell wall, but individual fragments extend from 0.05 to nearly 0.5 micron. The aggregates covered only a small fraction of the fields and most of the area was blank. Freeze-drying of the filtered effluent without centrifuging to concentrate the colloids and wash out the salts produced loose aggregates of dense particles. Salts are recognized only by their higher density because regular crystals had no time to form. Organic colloids may be present but are completely obscured by the particles of salts.

Some aggregation of small particles into clumps is produced by the method of preparation. Freezing concentrates solutes and suspended matter at crystal boundaries. When water is evaporated, some of the particles may be left at the top of a droplet as much as 300 microns above the carbon film. In a high vacuum, electrostatic charges will persist and cause the particles to stick together.

All of the freeze-dried preparations are remarkable for the absence of background debris. Comparison of the first three figures which were dried at room temperature with the last

Figure 1. Spore and attached cell wall showing fibers and a virus-like particle

Bar is 1 micron long in Figures 1 through 6

Figure 2. Cell wall fragments showing polygonal structure

Figure 3. Virus clump with cell fragments

Figure 4. Aggregates of freeze-dried cellular debris

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Figure 5. Cell wall and cell fragments
Freeze-dried

three which were freeze-dried clearly shows the difference. Viruses or other recognizable particles are apparently absent from most of the freeze-dried preparations because they are obscured by the excess of fragmentary colloids. Although quantitative estimates are difficult, the fragmentary colloids appear to be present in greater concentration than the viruses and other compact structures.

Clarification with lime or iron salts or filtration through 0.45-micron pore size membranes (Millipore) removed most of the colloidal material. Freeze-dried specimens showed principally blank fields with occasional aggregates of solids having the same loose structure found in other preparations. Both inorganic opaque specks and organic flakes were found. Some aggregates that formed in water which had passed through a 0.45-micron filter are much larger than the filter pore size.

Control tests for contamination were run on double distilled water which was treated in the same way as other capillary tube runs. Electron micrographs of the freeze-dried specimens showed hardly any detail. There were one or two small virus-like particles noticed in a careful scan of a dozen grids. A control slide of evaporated carbon on which no water was evaporated showed no structural details.

Conclusion

Colloidal matter is present in secondary effluent in size covering the range from at least 0.01 micron up to bacterial cell walls or flagella. There is undoubtedly also some larger material which might be classified as colloidal in one or two dimensions but which was removed by the initial filtration.

The dominant shape of the colloidal matter as fragments of very thin membranes accounts for the rapidity with which secondary effluents clog filters. The flat membranes act as leaves do in storm water and can be expected to clog fine pores as leaves clog storm sewer grids. Thin membranes provide the most efficient way to cover surface per unit of weight. An estimate of the concentration of organic colloidal carbon in secondary weights can be obtained from the data of Bishop *et al.* (1965, 1967). These investigators found that clarification removed about 4 mg. per liter of organic carbon when the total carbon was 12 to 15 mg. per liter. Removals up to 55 mg. per liter were obtained from richer secondary effluents. Even at these low concentrations, the colloids of

Figure 6. Partly disintegrated cell wall
Freeze-dried

biological origin are able to exert a profound effect on the performance of filters, granular adsorbents, and membranes.

The persistence of bacterial cell walls in the presence of the extracellular enzymes in activated sludge obviously derives from the resistance of the living cells to these same enzymes. It is reasonable to expect cell wall fragments to constitute the major fraction of cellular debris in a system in which microorganisms are growing and dying. Salton (1964) and Weidel and Pelzer (1964) refer to the ease of separating cell walls from other cellular material.

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